EXPERIMENTAL SECTION

Synthesis of BSA-NH_2/PNIPAAm conjugates

The BSA-NH_2/PNIPAAmconjugate was synthesized according to our previous methods [Huang, X.; et. al. *Nature Communications* **2013**, *4*, 2239]. First, cationized bovine serum albumin (BSA-NH_2) was synthesized by carbodiimide-activated conjugation of 1,6-diaminohexane to aspartic and glutamic acid residues on the external surface of the protein. For this, a solution of 1,6-diaminohexane (1.5 g, 12.9 mmol) was adjusted to pH 6.5 using 5 M HCl, and added dropwise to a stirred solution of the protein (200 mg, 2.98 μmol). The coupling reaction was initiated by adding N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC, 100 mg) immediately, and again (50 mg) after 5 h. The pH value was maintained at 6.5 using dilute HCl, and the solution was stirred for a further 6 h. The solution was then centrifuged to remove any precipitate and the supernatant was dialyzed (dialysis tubing 12–14 kDa MWCO) extensively against Milli-Q water.

End-capped mercaptothiazoline-activated PNIPAAm (M_n= 8,800 g mol^{-1}, 10 mg in 5 mL of water) was synthesized according to our previous reported methods [Huang, X.; et. al. *Nature Communications* **2013**, *4*, 2239], and added to a stirred solution of BSA-NH_2 (10 mg in 5 mL of PBS buffer at pH 8.0). The mixed solution was stirred for 12 h, and then purified by using a centrifugal filter (MWCO 50 kDa) to remove any unreacted PNIPAAm and salts. After freeze-drying, the BSA-NH_2/PNIPAAm conjugate was obtained. Based on MALDI-TOF and UV–vis spectroscopy measurements, there were *ca.* 3.3 PNIPAAm chains per BSA molecule.

Preparation of single compartmentalized proteinosomes

Proteinosomes were prepared by mixing an aqueous BSA-NH_2/PNIPAAm solution with 2-ethyl-1-hexanol followed by shaking the mixture by hand for 10 seconds. The samples were prepared at a constant aqueous/oil volume fraction (φ_w) of 0.06. Typically, 0.06 mL of aqueous BSA-NH_2/PNIPAAmin pH 8.5, sodium carbonate buffer were mixed with 1.0 mL of the oil. The

proteinosomes were then cross-linked in the continuous oil phase by addition of a NHS-PEG-containing cross-linker(PEG-bis(N-succinimidyl succinate (NHS-PEG9) ester or PEG-bis(N-succinimidyl succinate) disulfide ester (NHS-PEG16-DS)) for 12 h, which reacted with the free primary amine groups of BSA-NH_2.

Transfer of the cross-linked proteinosomes into water was achieved as follows. After 3 hours sedimentation (left to stand), the upper clear oil layer was discarded and 1 mL of 75% ethanol was added with a gentle shaking. Then the dispersion was dialyzed against 75% ethanol, and after 3 h dialysis, the dispersion was added dropwisely into 50 mL of Milli-Q water. Finally by dialyzing against water for at least 1 day to make sure all the oil was removed.

Preparation of multi-compartmentalized proteinosomes

Multi-compartmentalized proteinosomes were prepared based on a recursive Picking emulsion procedure. Typically, we used a 8.0 mg/mL aqueous dispersion of BSA-NH_2/PNIPAAm nanoconjugates to prepare a proteinosome stock solution comprising microcapsules with a mean size of ca. 15μm (proteinosome concentration; 1.0 mg/mL). We then mixed a certain amount of this stock solution with an aqueous solution of the BSA-NH_2/PNIPAAm nanoconjugates, keeping the total aqueous phase volume at 60 μL with the concentration of BSA-NH_2/PNIPAAm nanoconjuagtes equal to 1.0 mg/mL. After adding 1mL of 2-ethyl-1-hexanol into the solution, the mixture was shaken by hand for 10 seconds, and a proteinosome-in-proteinosome structure obtained. The nested proteinosomes were cross-linked with a NHS-PEG-containing cross-linker, and the transferred into aqueous solution.

Encapsulating the two-tier proteinosome structure into a larger proteinosome to produce a three-tiered microarchitecture was undertaken by repeating the above procedures with appropriate modifications in the concentration of the BSA-NH_2/PNIPAAm nanoconjugates to generate appropriately sized host proteinosomes. Typically, the sequence order of concentrations of BSA-NH_2/PNIPAAm nanoconjugates used was 8.0 mg/mL, 1.0 mg/mL and 0.5 mg/mL.

Sub-compartment encapsulation of enzymes into hierarchically ordered

The above procedures were adopted except that the enzymes were added to the aqueous BSA-NH_2/PNIPAAm solution prior to mixing with the oil phase. For example, the targeted loading of a three-tier proteinosome with different fluorescence-labeled enzymes was undertaken by first encapsulating fluorescein isothiocyanate-labeled glucose oxidase (FITC-GO, green) into small proteinosomes, which were subsequently used as the first guest microcapsules. Rhodamine B isothiocyanate-labeled glucose amylase (RBITC-GA, red) together with the pre-formed FITC-GO-containing proteinosomes were added to an aqueous solution of the BSA-NH_2/PNIPAAm nanoconjugates (final concentration, 1.0 mg/mL) and the two-tier proteinosomes cross-linked and transferred to water as described above. Finally, the procedure was repeated by mixing the two-tier FITC-GO/RBITC-GA proteinosomes with DyLight 405-labeled horseradish peroxidase (DL405-HRP, blue) and a solution of BSA-NH_2/PNIPAAm nanoconjuagtes (0.5 mg/mL in the mixed solution) to produce a three-tier triple proteinosome with spatially organized enzymes.

Programmed release ofRBITC-Dextran and DNA from two-tier proteinosomes

DNA (salmon testes, Sigma, ~ 2000b.p) was encapsulated into the interior of single-level proteinosomes by adding aqueous DNA (20 μL, 2.0 mg/mL) to 40 μL of an aqueous solution of BSA-NH_2/PNIPAAm nanoconjugates (12 mg/mL), followed by addition of 1.0 mL of 2-ethyl-1-hexanol. The proteinosomes were then cross-linked with NHS-PEG9 ester, and transferred into water as described above. The DNA loaded proteinosomes were mixed with an aqueous solution of RBITC-dextranand BSA-NH_2/PNIPAAm nanoconjugates (final concentration, 1.0 mg/mL), followed by addition of 1.0 mL of 2-ethyl-1-hexanol and NHS-PEG9 ester to cross-link the host proteinosome, and transfer into water. Under these conditions, the two-tiered micro-architecture consisted of DNA and dextran encapsulated specifically within the guest and in proteinosomes, respectively, and comprised

non-disulfide membranes throughout. Similar procedures were used to capture DNA and dextran in the guest and host compartments, but the membranes of the guest and host proteinosomes were cross-linked using different reagents (NHS-PEG9 esterorNHS-PEG16-DS) to produce two-tiered structures comprising different membrane chemistries.

For the two-tier proteinosomes cross-linked with only NHS-PEG9 ester, release of encapsulated DNA and RBITC-dextran was induced by protease. An aqueous dispersion of the DNA/RBITC-dextran-proteinosome-in-proteinosome was mixed with 20 mL of a buffered protease solution (*Streptomyces griseus,* Sigma, ≥3.5units/mg solid, M_w ~50kDa; pH 7.4 buffer) at a concentration of 0.01 mg/mL. Aliquots (2 mL) of the suspension were removed at various time intervals, filtered to remove any intact proteinosomes, and the concentration of released SYBR green I-stained DNA or RBTIC-dextran in the bulk phase determined by measuring changes in fluorescence intensity at 522 nm for DNA, and 580 nm for RBITC-dextran. This procedure was also used to determine the release of encapsulated components from the NHS-PEG9/NHS-PEG16-DS cross-linked proteinosome-in-proteinosomes. In this case, release was first triggered by addition of tris(2-carboxyethyl)phosphine (TCEP) (2.5 mM, in pH 8.0 PBS) for 20-40 minutes, followed by addition of 1mg/mLof protease.

Synthesis of fluorescence-labeled proteins

Fluorescein isothiocyanate (FITC)-labeled glucose oxidase (FITC-GO) was synthesized by dissolving a dried powder of the protein (5.0 mg) in 2.0 mL of pH 8.5 sodium carbonate buffer solution (100 mM), followed by dropwise addition of 50 μLDMSO solution of FITC (1.0 mg/mL). The solution was stirred at room temperature for 5 h, purified by dialyzing against Milli-Q water, and freeze-dried. Rhodamine B isothiocyanate (RBITC)-labeled glucose amylase, and DyLight 405-labeled horseradish peroxidase (DL405-HRP) were prepared by the same procedure using a DMSO solution of RBITC (1.0 mg/mL) or aqueous solution of DL405 (1.0 mg/mL).

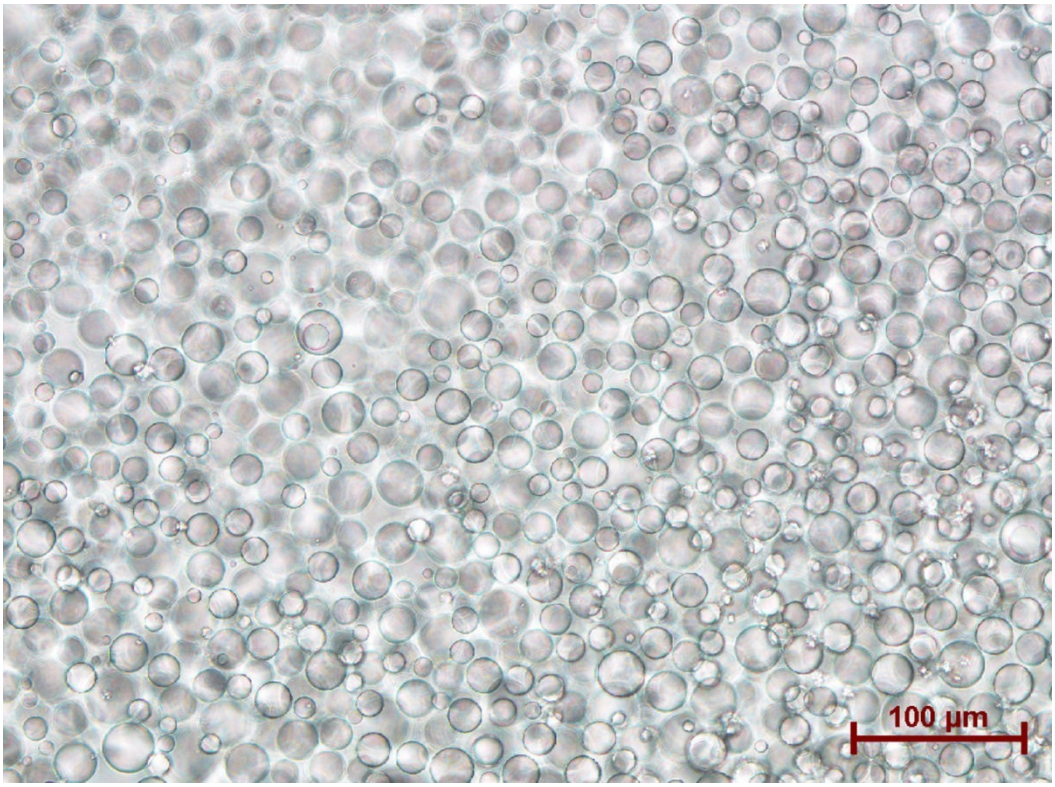

Figure S1. Optical microscopy image of single-chambered BSA-NH_2/PNIPAAmproteinosomes dispersed in 2-ethyl-1-hexanol. The sample was prepared under vigorous shaking at a φ_w value of 0.06, and using a BSA-NH_2/PNIPAAm concentration of 8.0 mg/mL.

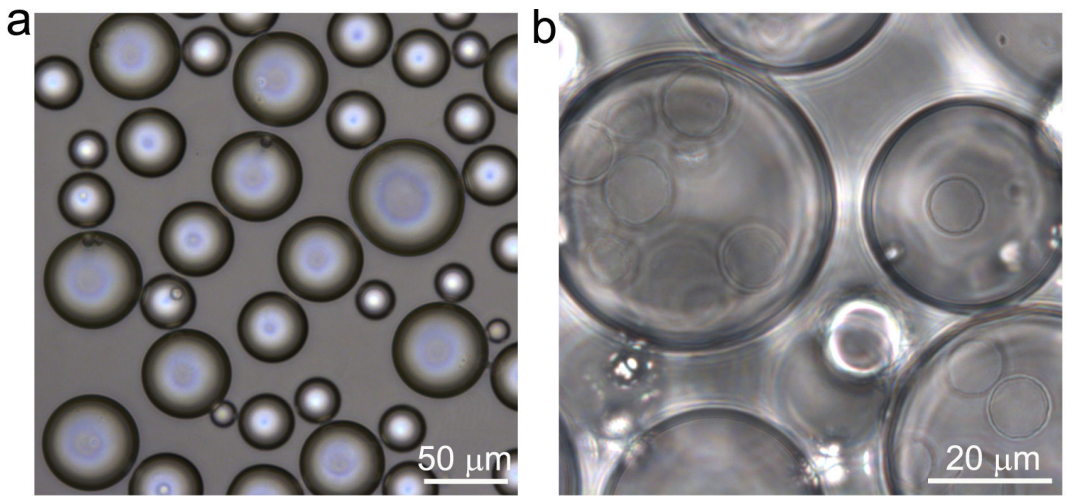

Figure S2. Optical microscopy images of two-tiered BSA-NH_2/PNIPAAm proteinosomes dispersed in oil (a), and after partial drying in air (b).

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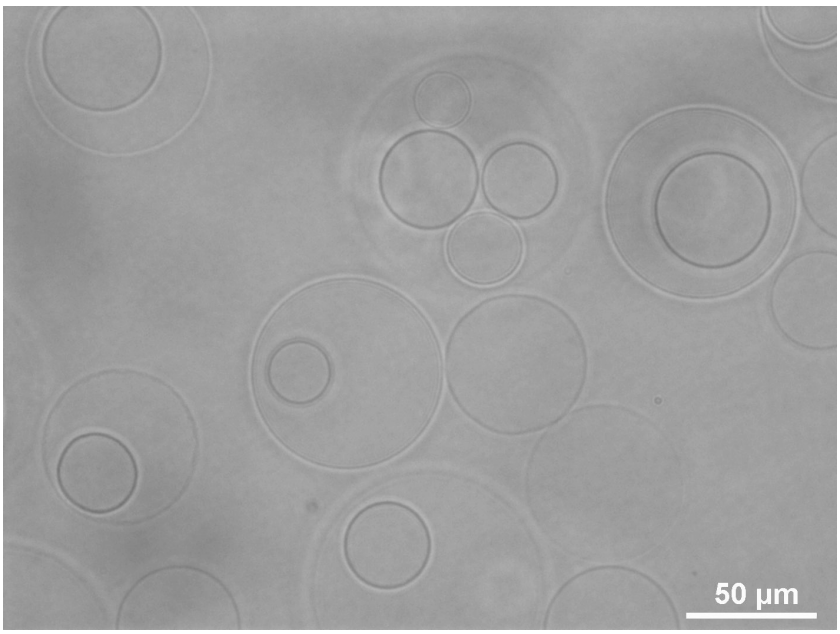

Figure S3. Optical microscopy image of two-level BSA-NH_2/PNIPAAmproteinosomes prepared at a guest proteinosome: protein-polymer nanoconjugate mass ratios of 4 : 10.

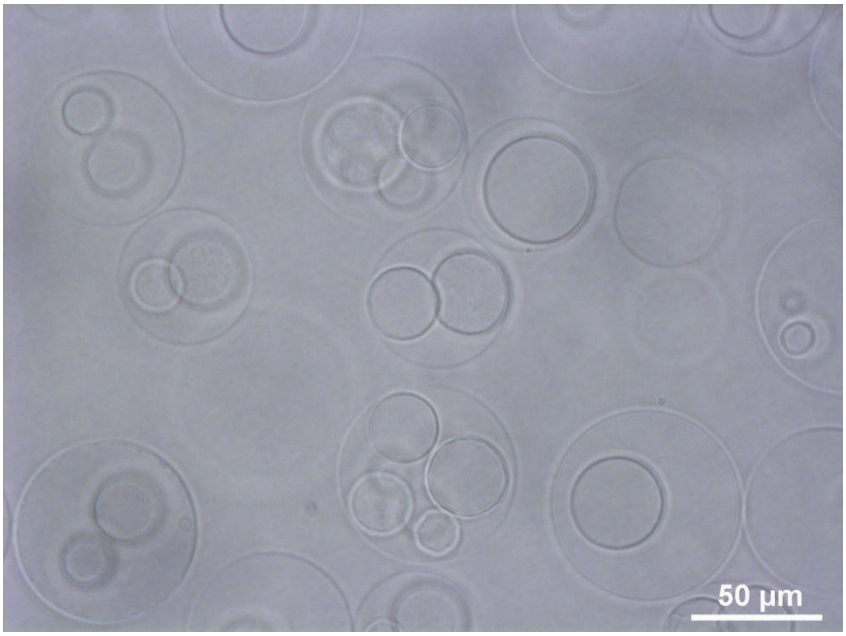

Figure S4. Optical microscopy image of two-level BSA-NH_2/PNIPAAm proteinosomes prepared at a guest proteinosome: protein-polymer nanoconjugate mass ratios of 8 : 10.

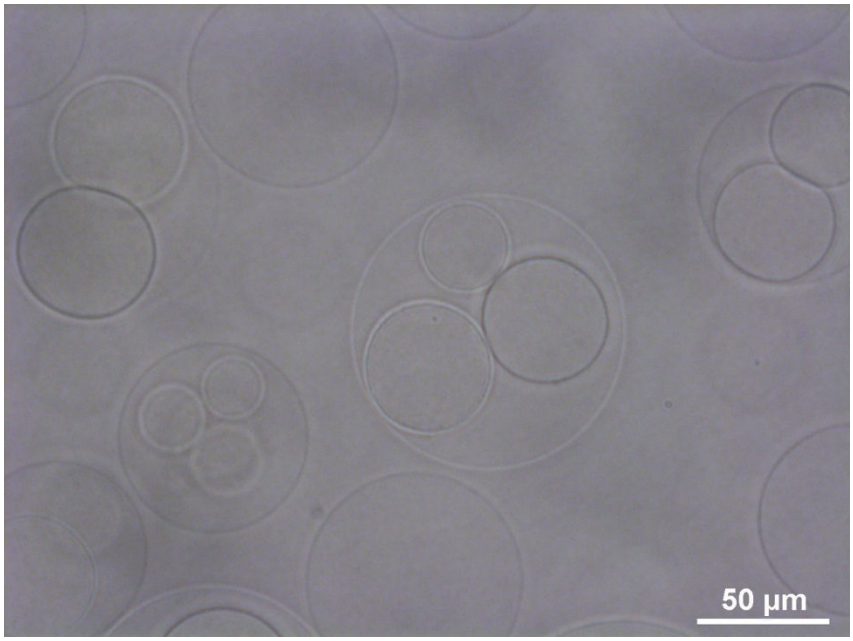

Figure S5. Optical microscopy image of two-level BSA-NH_2/PNIPAAm proteinosomes prepared at a guest proteinosome: protein-polymer nanoconjugate mass ratios of 12 : 10.

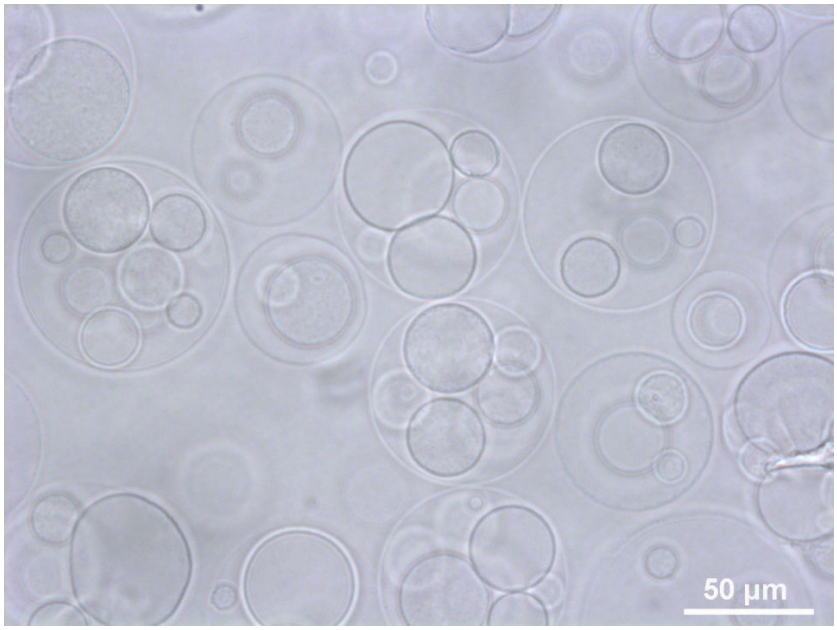

Figure S6. Optical microscopy image of two-level BSA-NH_2/PNIPAAm proteinosomes prepared at a guest proteinosome: protein-polymer nanoconjugate mass ratios of 30: 10.

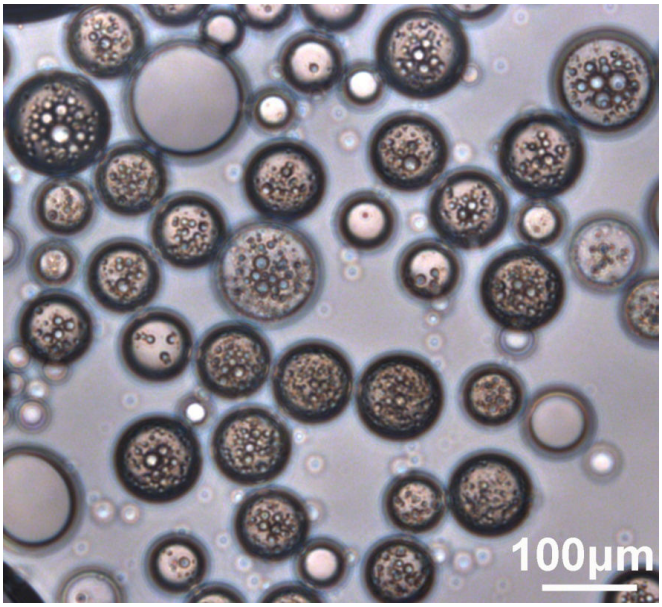

Figure S7. Optical microscopy image showing multi-compartmentalized BSA-NH_2/PNIPAAm proteinosomes in 2-ethyl-1-hexanol prepared by encapsulating 8-15 µm-sized guest proteinosomes into individual host proteinosome 80-100 µm in diameter. Tens of proteinosomes are encapsulated within each host microstructure.

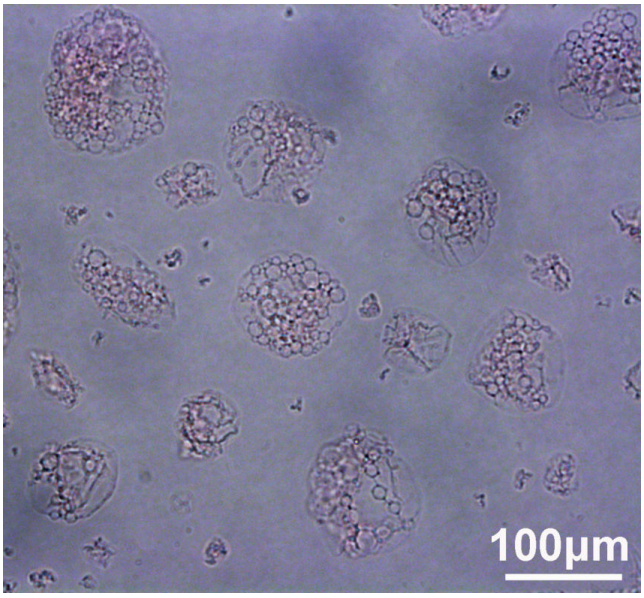

Figure S8. Optical microscopy image of multi-compartmentalized BSA-NH_2/PNIPAAm proteinosomes in 2-ethyl-1-hexanol phase after drying. The samples were prepared by encapsulating. 8-15 µm-sized guest proteinosomes into individual host proteinosome 80-100 µm in diameter. Tens of proteinosomes are encapsulated within each host microstructure.

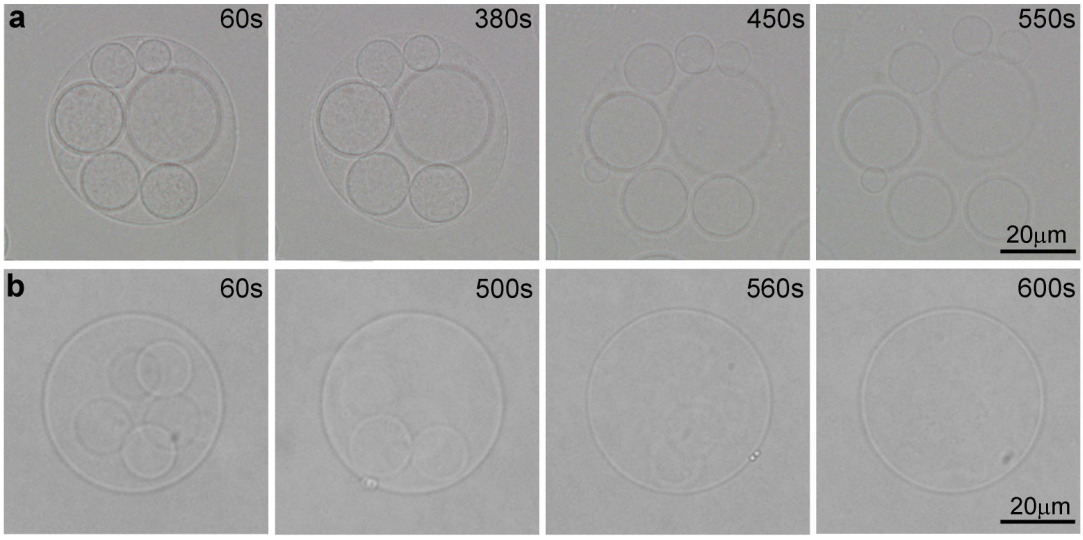

Figure S9. (a,b) Time-dependent series showing optical microscopy images of two-tiered proteinosomes with host and guest membranes cross-linked respectively by NHS-PEG16-DS or NHS-PEG9 ester (a), or NHS-PEG9 ester or NHS-PEG16-DS, respectively (b), and after addition of TCEP (5 mM, pH 8.0). (a) Complete disassembly of the host proteinosome occurs after 550 s whilst the guest proteinosomes remain intact and slowly disperse into the external solution. (b) Complete disassembly of the guest proteinosome membranes occurs after 600 s leaving an intact host proteinosome.